

Use of the Caco-2 Cell Model to Assess the Relative Lead-chelating Ability of Diastereoisomers of 2,3-Dimercaptosuccinic Acid

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The purpose of this study was to examine the mechanisms of lead (Pb) uptake by human intestinal cells and to compare the intestinal transport and relative lead-chelating ability of two diastereoisomeric forms (i.e., *meso* and racemic) of 2,3-dimercaptosuccinic acid (DMSA). The model used was the human adenocarcinoma (Caco-2) cell monolayer. The Caco-2 cells were cultured in flasks for examination of cellular uptake of lead and subsequent chelation of the lead by the DMSA isomers. For assessment of the comparative intestinal transport of the diastereoisomers, the Caco-2 cells were cultured on semipermeable supports. The effects of *N*-ethylmaleimide and 1,25-dihydroxyvitamin D₃ (vitamin D₃) on the uptake of lead by the Caco-2 monolayer were examined to determine the contributions of sulfhydryl-binding and calcium-binding protein, respectively, to the lead uptake process. Analysis of lead was performed using both macro- and micro-proton-induced X-ray emission (PIXE), and DMSA was measured spectrophotometrically following derivatization with 5,5'-dithiobis-2-nitrobenzoic acid. Results from micro-PIXE imaging suggest that lead is bound on the surface of the cell, and that sulfhydryl binding may be an important step in the uptake of lead by the Caco-2 cells. Macro-PIXE results indicate that the racemic form of DMSA may be more effective in chelating lead from within the cell. Comparison of the transport of the two DMSA diastereoisomers indicates that the racemic form is transported across the Caco-2 monolayer more readily than the *meso* form. **Key words:** Caco-2, 2,3-dimercaptosuccinic acid, lead, proton-induced X-ray emission. *Environ Health Perspect* 107:111–115 (1999). [Online 11 January 1999]

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Lead intoxication is a serious health problem. Blood levels of lead as low as 10–15 µg/dl, and possibly lower, are linked to undesirable developmental effects in human fetuses and children (1). These effects include impaired neurobehavioral development and other possible effects on early development and growth.

Currently the only orally administered treatment for lead poisoning is *meso*-2,3-dimercaptosuccinic acid (*meso*-DMSA), which is available commercially as Succimer (Chemet, Bock Pharmacal Co., St. Louis, MO). This compound was approved by the Food and Drug Administration in 1991 for treatment of childhood lead intoxication when blood lead levels exceed 45 µg/dl. Since that time, there has been an increasing use of *meso*-DMSA for the treatment of children with blood lead levels as low as 20 µg/dl (2).

Despite its increasing clinical use, *meso*-DMSA has several disadvantages. It is virtually insoluble in acidic solution and only slightly soluble in water. These properties may reduce the gastrointestinal absorption of the drug and result in the need for a comparatively higher dose of *meso*-DMSA to maintain an effective blood level for chelation therapy. In fact, studies have found that only 20.6% of the administered dose of *meso*-DMSA is found in the urine as total *meso*-DMSA 14 hr after the drug is given orally (3,4). In addition, two unpublished

bioavailability studies conducted at McNeil Laboratories indicate that the oral bioavailability of the commercial *meso*-DMSA formula was only about 25% (5). Another disadvantage of *meso*-DMSA is that it reportedly removes lead only from the extracellular fluid of plasma (6), and thus it may be unable to mobilize lead from within the brain, bone marrow, or other soft tissues where lead can deposit.

DMSA exists in two diastereoisomeric forms, *meso* and racemic (*dl*-). The *dl*-DMSA form is soluble in water, strongly acidic solutions, and in nonaqueous solvents such as ethyl ether (7). On this basis it was proposed that *dl*-DMSA will be absorbed across the intestinal epithelium more efficiently than the *meso* diastereoisomer upon oral administration and that it may be a more effective chelator for the treatment of lead poisoning (8).

The efficacy of *dl*-DMSA in the treatment of lead poisoning has not been studied in either human subjects or animals. However, *in vivo* studies with *dl*-DMSA showed that it was more effective than *meso*-DMSA in reducing mortality and increasing the excretion of mercury and cadmium from the body in cases of acute poisoning (9–11). The results of these *in vivo* studies with mercury and cadmium have been rationalized recently on the basis of higher formation constants for the complexes of *dl*-DMSA

with cadmium and mercury at physiological pH (12).

To help determine whether *dl*-DMSA might be more effective than *meso*-DMSA in the treatment of lead poisoning, we used the Caco-2 monolayer cell culture model to elucidate the comparative epithelial transfer of the two forms of DMSA. The suitability of this model for elucidating the transport pathways of a wide variety of molecules, including peptides, proteins, and metals (e.g., calcium and cadmium), is well established (13,14). The advantages of this model include 1) rapid assessment of absorption and metabolism of molecules, 2) independent access to both the apical (AP) and basolateral (BL) sides of the cell monolayer to permit monitoring both the appearance and disappearance of solutes, 3) the use of human cells as opposed to tissues from animal sources, 4) greater cell viability compared with isolated or perfused systems, and 5) better reproducibility (15). Recent studies (15–17) have shown good correlation between data from this model and other well-established *in vitro* and *in situ* models traditionally used for conducting such absorption studies (16,18).

The uptake and internalization of lead has been shown to mimic that of calcium in chick and bovine intestinal cells (19). Calcium uptake involves an initial internalization step followed by intracellular binding to calcium-binding protein (CaBP) (20), which is thought to act as a “ferry” for transporting calcium across the intracellular space. Transcription of CaBP is enhanced by the presence of vitamin D₃ in the nucleus. When blood calcium levels fall, the kidney is stimulated by parathyroid hormone to synthesize more vitamin D₃ from existing vitamin D (19). Increased levels of vitamin D₃ cause the gastrointestinal cells to produce more CaBP,

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ultimately resulting in increased uptake of calcium from the gastrointestinal tract (19).

A micro-proton-induced X-ray emission (micro-PIXE) technique (21) was used to assess the uptake of lead by the Caco-2 cells. This was accomplished by measuring the concentrations of lead, zinc, and other essential elements in cells exposed to lead solutions. The relative ability of the two forms of DMSA to mobilize lead, zinc, and other essential elements from inside the cells was assessed using macro-PIXE (22). This technique provides information on the concentrations of elements above atomic number 11 and is the only analytical method that can measure 20–30 elements nondestructively in a single small sample (≤ 5 mg) with a detection limit of 1–5 ppm (dry weight).

The goal of the present study was to examine the behavior of the two isomers of DMSA in a tissue culture model by using PIXE to quantitate lead within cells treated with both lead and *dl*- or *meso*-DMSA. In addition to quantitative assessment of lead in the Caco-2 cells, micro-PIXE was used to qualitatively assess the location of lead within the cells.

Materials and Methods

Cell culture. Caco-2 cells (American Type Culture Collection, Rockville, MD) were cultured in T-75 flasks as described previously (15). The protocol for seeding the cells in Transwell cell culture inserts (Costar, Cambridge, MA) was as described earlier

(15), with the following modifications. The 12-well tissue culture clusters were prepared in the laminar flow hood by pipetting 1.5 ml of complete medium (38°C) into each well. A Transwell insert was placed in each well using a pair of sterile forceps. Aliquots of a 7×10^4 cell suspension (0.5 ml) were then pipetted into the apical chamber of each Transwell insert, and the plates were transferred to the incubator.

Cell viability studies. Before the lead uptake and DMSA transport experiments, we conducted cytotoxicity assays to ensure that the viability of the cells was not compromised in the presence of the experimental compounds at the concentrations to be tested. This was accomplished by comparing the amount of lactate dehydrogenase (LDH; assay kit DG1340K, Sigma, St. Louis, MO) released by the cells after treatment with a range of concentrations of lead or the DMSA diastereoisomers with the amount of LDH released by a control exposed to serum-free medium. LDH, an enzyme released from within a cell when it dies, is a good indicator of the damage to a population of cells caused by experimental treatment. The toxicities of vitamin D₃ and *N*-ethylmaleimide (NEM) were determined in previous studies involving Caco-2 cells (23), and the experimental concentrations from these previous experiments were adopted for the present study.

The cytotoxicity studies were performed in 96-well tissue culture plates (Falcon/Becton Dickinson Labware, Lincoln Park, NJ). For the transport studies, eight-well strip tissue culture inserts (Nalge-Nunc International, Naperville, IL; miniature Transwells) were used to mimic the conditions of the actual experiment. The cells were seeded (4×10^4 cells/ml) into the 96-well culture plates with a multichannel pipet. In the case of the cytotoxicity studies for the uptake experiments, the cell suspension was seeded in 200- μ l

aliquots directly into the wells of the 96-well plate. For the DMSA transport cytotoxicity studies, 100 μ l of complete medium (38°C) was added to each well. The 8-well strip inserts were then placed into the plate, and 50- μ l aliquots of cell suspension were pipetted into each apical chamber. The cells were then placed in the incubator overnight to allow them to attach to their respective substrates.

On the day after seeding, the medium was removed from the wells using a multi-channel pipet. The medium was then replaced with serum-free medium that contained various concentrations of the experimental compound to be tested. This solution was allowed to remain in contact with the cells for 30 min, after which it was removed using a 200- μ l pipet and placed in plastic microcentrifuge tubes for storage at 4°C until the time of assay. All samples were assayed for LDH within 24 hr.

The assay for LDH release was performed according to the instructions included with the Sigma LDH measurement kit with the following modifications. The kinetic assay technique was abandoned in favor of a less complicated and equally informative end point assay. To initiate the analysis, 100 μ l of sample from the 96-well assay was added to a cuvette containing 2.5 ml of the reagents from the LDH measurement kit. This mixture was allowed to equilibrate at room temperature for 20 min and then assayed spectrophotometrically at 340 nm (Beckman Instruments, Fullerton, CA) as detailed in the instructions.

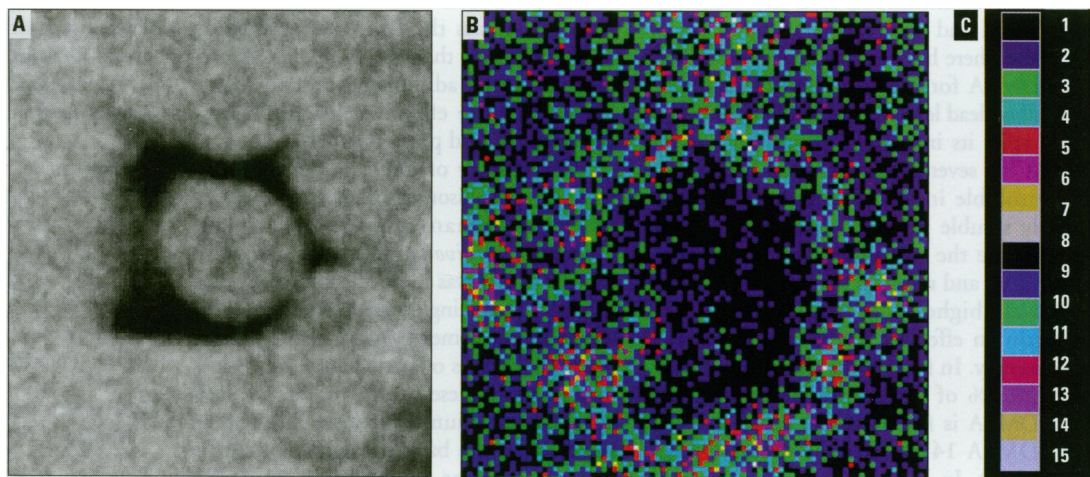
Optimization of lead concentrations and exposure times. Before performing the lead uptake experiments, we assessed the optimum conditions for lead uptake by incubating each flask of cells with 10 ml of serum-free medium (SFM) containing different concentrations of lead (PbNO_3 , 99.9% pure; J.T. Baker, Phillipsburg, NJ) for various exposure times (see Table 1).

Table 1. Summary of results from the lead uptake optimization experiments

Exposure time (hr)	Pb (μ M)	Pb uptake \pm SE (ng/cm ²)	Lactate dehydrogenase (% total \pm SE)
0.5	500	$1.1 \times 10^5 \pm 1.2 \times 10^4$	0.21 ± 0.21
2	80	22.3 ± 6.9	10.6 ± 3.6
4	50	553.2 ± 122.2	58.6 ± 2.9

SE, standard error.

Figure 1. (A) Light microscopy image of the area scanned by the PIXE beam. (B) Micro-PIXE image of the Caco-2 cell showing high lead concentrations in the cell membrane. (C) Scale representing the counts per color in the images.



The medium from each flask was then assayed for LDH as described previously. The optimum lead concentration was considered to be the one that resulted in the highest intracellular lead concentration and did not induce cell death as indicated by the LDH assay.

Micro-PIXE measurement of lead uptake by the Caco-2 cells. For the micro-PIXE experiments, the Caco-2 cells were seeded onto 1-cm diameter microscope cover slips that had been placed in 12-well tissue culture plates, at a seeding density of approximately 3×10^6 cells/ml. The cells were cultured for 1–2 days to allow them to attach to the cover slips. The cells in the vitamin D₃ experimental group were treated with 100 nM vitamin D₃ 24 hr before exposure to lead. The NEM treatment group was exposed to 500 μ M NEM for 20 min immediately before the lead exposure.

To initiate the uptake experiments, the medium was aspirated from the wells and replaced with 2 ml of SFM containing 500 μ M lead. After 30 min of exposure to the lead, the SFM was removed and the cell layers gently rinsed once with SFM. Following the rinsing step, the treatments were added to the wells. A treatment group consisted of three wells. The four treatment groups were 1) 500 μ M lead only, 2) cells pretreated with 100 nM vitamin D₃, then 500 μ M lead, 3) cells pretreated with 500 μ M NEM, then 500 μ M lead, and 4) cells pretreated with both vitamin D₃ (100 nM) and NEM (500 μ M), followed by exposure to 500 μ M lead.

After 30 min of exposure to the 500 μ M lead, the cells were gently rinsed with

SFM. Forceps were used to remove the cover slips from the wells. The cover slips, with their attached cell layer, were then coated by dipping them into a water bath on the surface of which had been floated a thin layer of carbon. The carbon layer was necessary to disperse charge throughout the samples and thereby reduce background. The coated samples were placed in a vacuum chamber attached to the micro-PIXE apparatus and allowed to dry overnight. Cells were examined visually using light microscopy to position them directly in the path of the micro-PIXE beam. The cells were then subjected to analysis by micro-PIXE and the resulting images stored on computer disk for future examination.

Chelation of lead from the Caco-2 cells by the DMSA diastereoisomers. We assessed the comparative chelating ability of the two DMSA diastereoisomers by first exposing a flask of cells to 500 μ M lead for 30 min. The lead-containing medium was then aspirated and the cells were rinsed once with SFM. After rinsing, 10 ml of SFM alone, or SFM containing 50 μ M *meso*-DMSA or 50 μ M *dl*-DMSA was added to each flask. After 15 min of exposure to the DMSA diastereoisomers, the cell monolayers from each experiment were washed once with SFM alone and then with a $1 \times$ trypsin-EDTA solution to chelate any remaining extracellular lead and to remove the cells from the substrate. After 8–10 min of trypsinization, the cells were scraped from the substrate with a cell lifter and centrifuged (Model TJ-6, Beckman Instruments) for 10 min at 400g. The supernatant was then aspirated. The resulting cell pellet was transferred by pipet to a round

piece of filter paper (6 mm in diameter) that had been placed on a piece of mylar (2.5 μ m) stretched over an X-ray fluorescence cup (3.2 cm in diameter; Chemplex Industries, Tuckahoe, NY). The pellet was allowed to dry overnight and was then covered with another sheet of mylar. This preparation was then analyzed using macro-PIXE. To normalize the data for possible differences in cell density, we measured the lead-to-iron ratio for each treatment group.

Transport of the DMSA diastereoisomers across the Caco-2 monolayer. Caco-2 cells were routinely seeded into Transwell 12-well plate inserts as described previously (15). To initiate the experiments, the AP fluid was replaced with transport buffer alone (Hanks' Balanced Salt Solution with 5.6 mM glucose and 20 mM HEPES, pH 7.2, 38°C), or transport buffer containing 50 μ M *meso*- or *dl*-DMSA. The basolateral medium was replaced with transport buffer. After 15 min of exposure to the DMSA diastereoisomers, medium from the basolateral chamber was sampled. The samples were directly transferred to test tubes and assayed spectrophotometrically for DMSA content using Ellman's reagent (24) to quantitate the amount of DMSA transported across the monolayer.

Results and Discussion

Cell viability studies. The maximum DMSA concentration tolerated by the Caco-2 cell monolayer, without significant cell death (determined by LDH release), when compared with the control, was 50 μ M for *meso*-DMSA and between 25 and 50 μ M for *dl*-DMSA (data not shown). This is consistent

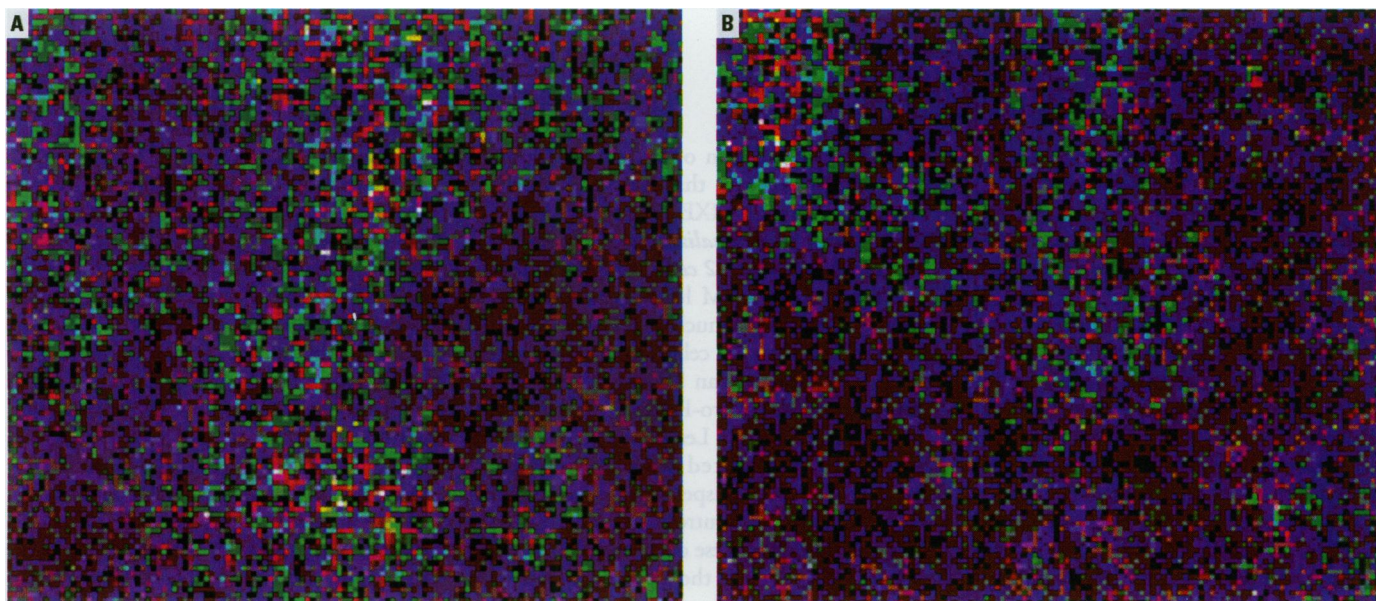


Figure 2. (A) Micro-PIXE image of lead concentration in Caco-2 cells pretreated with vitamin D₃. (B) Lead concentration in untreated Caco-2 cells. Each color represents 2 counts on the scale depicted in Figure 1C.

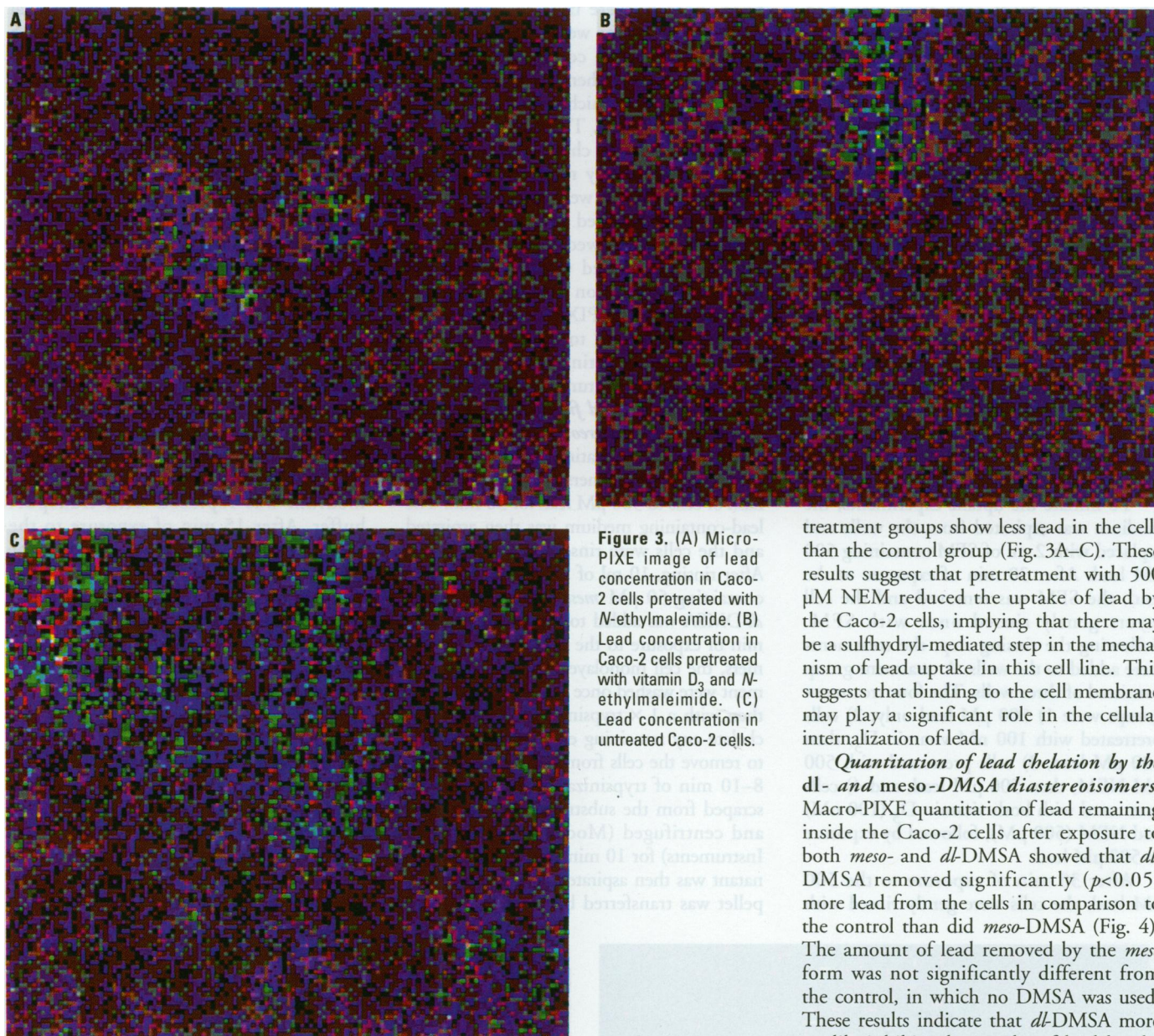


Figure 3. (A) Micro-PIXE image of lead concentration in Caco-2 cells pretreated with *N*-ethylmaleimide. (B) Lead concentration in Caco-2 cells pretreated with vitamin D₃ and *N*-ethylmaleimide. (C) Lead concentration in untreated Caco-2 cells.

with the findings of Aposhian and Aposhian (6), who found *dl*-DMSA to have a lower LD₅₀ than *meso*-DMSA in mice.

Optimization of lead concentrations and exposure times. The optimum lead concentration and exposure time was 500 μ M for 30 min. These conditions resulted in the least amount of cell death and the highest cellular lead concentrations (see Table 1). The magnitude of the cellular lead concentration was an important consideration for the study, due to the fact that it was difficult to quantitatively assay low lead concentrations with macro-PIXE. Quantitating lead in small samples (<5 mg) proved to be problematic. Initial attempts to measure lead in cells collected from a 12-well tissue culture plate were unsuccessful, and we decided to use an entire flask of

cells for each trial in order to concentrate the amount of lead that would be in the path of the macro-PIXE beam.

Micro-PIXE visualization of the uptake of lead by the Caco-2 cells. After 30 min of exposure to 500 μ M lead, the cell membrane contained a much higher concentration of lead than the cell interior or the surrounding fluid, as can be seen in the light microscopy and micro-PIXE image (Fig. 1A and B, respectively). Lead levels in the cells that were pretreated with vitamin D₃ appear, by visual inspection, to be higher than those of the control (lead only) group (Fig. 2A and B). These data suggest that vitamin D₃ enhanced the uptake of lead by the Caco-2 cells, although the lead concentration cannot be quantitated accurately. Both the NEM and the vitamin D₃/NEM

treatment groups show less lead in the cells than the control group (Fig. 3A–C). These results suggest that pretreatment with 500 μ M NEM reduced the uptake of lead by the Caco-2 cells, implying that there may be a sulfhydryl-mediated step in the mechanism of lead uptake in this cell line. This suggests that binding to the cell membrane may play a significant role in the cellular internalization of lead.

Quantitation of lead chelation by the *dl*- and *meso*-DMSA diastereoisomers. Macro-PIXE quantitation of lead remaining inside the Caco-2 cells after exposure to both *meso*- and *dl*-DMSA showed that *dl*-DMSA removed significantly ($p < 0.05$) more lead from the cells in comparison to the control than did *meso*-DMSA (Fig. 4). The amount of lead removed by the *meso* form was not significantly different from the control, in which no DMSA was used. These results indicate that *dl*-DMSA more readily inhibits the uptake of lead by the Caco-2 cells.

Transport of *dl*- and *meso*-DMSA across the Caco-2 monolayer. Fang et al. (12) hypothesized, based upon its higher solubility in gastric fluids and its more lipophilic nature, that the *dl*-DMSA would more readily penetrate the cell membrane and therefore the intestinal epithelium. In fact, significantly larger amounts of 50 μ M *dl*-DMSA were transported across the monolayer in comparison to the *meso*-DMSA (Fig. 5). The amount of *meso*-DMSA transported across the monolayer was not significantly different from background. These findings are consistent with the predictions pertaining to the relative lipophilicity of *dl*-DMSA compared with that of *meso*-DMSA made by Fang and Fernando (8).

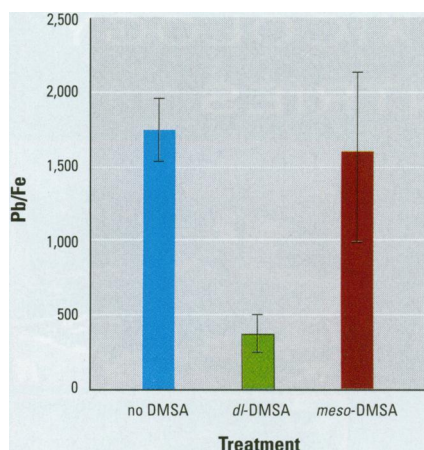


Figure 4. Lead-to-iron ratios in Caco-2 cells after a 15-min exposure to *dl*- and *meso*-dimercaptosuccinic acid (DMSA). Error bars indicate standard error.

Conclusions

The PIXE technique is an effective method for measuring lead, and possibly other metals, in a cell culture system. To achieve maximal intracellular lead concentrations that can be readily quantitated using the macro-PIXE technique without compromising the viability of the cells, we recommend that the cells be exposed to high concentrations of lead for relatively short periods of time. After 30 min of exposure of the Caco-2 cells to 500 μ M lead, it appeared that the majority of the lead was localized at the cell surface, indicating that there was an initial binding to the cell surface. The fact that pretreatment of the cells with NEM, a known sulfhydryl group blocker, negated this effect suggests that binding to sulfhydryl groups is involved in this process. The observation that the cells pretreated with vitamin D₃ followed by NEM also showed little lead uptake suggests that the initial surface binding of the lead is an important step in the subsequent internalization of the lead. The transport of *dl*-DMSA across the cell monolayer was significantly greater than that of the *meso* isomer. Based upon its superior

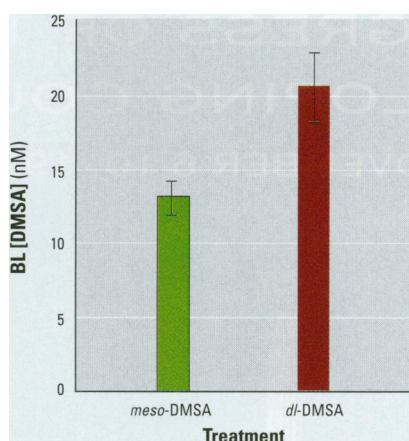


Figure 5. Comparative transport of *dl*- and *meso*-dimercaptosuccinic acid (DMSA) across the Caco-2 monolayer. Error bars indicate standard deviation.

ability to remove lead from within the Caco-2 cells exposed to it, the racemic form of DMSA appears to have the potential to be a more effective chelator of lead than the currently used *meso* form. Further studies to validate the utility of the racemic isomer of DMSA are warranted.

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